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A combined molecular typing approach does not discriminate *Legionella pneumophila* serogroup 1 strains of a predominant sequence-based type in Palermo, Italy

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Summary The sequence-based type 1,4,3,1,1,1 of *Legionella pneumophila* sg.1 is predominant in the Palermo city environment since several years. In this study, extended sequence-based typing and pulsed field gel electrophoresis were used in a combined approach in the aim to enhance discriminatory power of the molecular typing procedures. However, probably due to a common environmental reservoir and genetic stability, most of the strains circulating in the geographic area under study belong to the same clone and are, consequently, indistinguishable by molecular typing. Investigations of clinical cases and tracing to their environmental source require caution and support from sound epidemiological data.

Introduction

Legionellae are normal inhabitants of the fresh water environment, but are pathogenic for humans, causing serious disease in immunocompromised subjects. Legionellosis invariably originates from environmental sources, and may occur as either dramatic outbreaks or, more often, apparently sporadic case of healthcare- or community-associated respiratory illness.

Epidemiological investigations are usually performed when new cases occur, in order to locate the source and the extent of a possible outbreak, adopt targeted preventive measures and identify

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any legal responsibilities. Due to the ubiquitous prevalence of legionellae in water supply systems, strains from patients and environmental sources must be compared by molecular typing techniques to confirm or exclude a link with the suspected environmental reservoir [1]. Moreover, risk management of some critical water systems, such as those supplying healthcare facilities, could also be helpfully integrated by information pertaining differential susceptibility to chlorine treatment of some *Legionella pneumophila* strains [2]. Additionally, specific host-related risk factors have been recently associated with legionellosis cases due to some well-characterized endemic clones [3].

A sequence-based typing (SBT) using the sequences of six bacterial genes (*fla*A, *pil*E, *asd*, *mip*, *momp*S, and *pro*A), was described in 2005 by Gaia et al. [4], and proposed as a new gold standard for the epidemiological typing of *L. pneumophila* sg.1. As *L. pneumophila* sg.1 types are identified by numerical profiles, SBT shows excellent reproducibility and, when compared with other typing procedures, requires less subjectivity in interpretation of the results. However, some SBT profiles are proving to be clonally disseminated in wide geographical areas [3]. Consequently, any apparent epidemiological link between clinical and environmental isolates must be interpreted with caution. To enhance the discriminatory power of the standard sequence-based scheme, the additional use of *neu*A as a seventh allele for SBT or combined typing approaches have been recently proposed [5—7].

In a recently published study, we described the very frequent isolation in Palermo, Italy, of strains belonging to an identical AFLP type and to the SB type 1,4,3,1,1,1 [8]. This latter is also the most common type in many parts of the world [7,9—11]. Because of the apparently prominent role in our geographic area of *L. pneumophila* sg.1 strains attributable to this SB type, we evaluated the possibility of obtaining an improved discrimination by combining the extended SBT scheme with the additional use of the *neu*A gene and pulsed-field gel electrophoresis (PFGE) [12]. The objective was to assess the reliability of such a combined molecular approach as a support to epidemiological investigations on legionellosis in our geographic area.

Materials and methods

One hundred and eleven strains of *L. pneumophila* sg.1 were isolated in our laboratory between 2003 and 2008 from the sputum of 2 hospitalized patients and from 18 different environmental sites in Palermo and northern Sicily (hospitals, hotels, private houses, a dental surgery and an aqueduct) (Table 1). For the purpose of the study, strains belonging to different standard six-gene SB types or isolated from different sites and/or in different years were considered as probably epidemiologically unrelated.

Table 1 Sequence-based genotypes of *L. pneumophila* identified in northern Sicily, time and sites of their isolation, and number of the isolated strains.

Genotype *fla*A, *pil*E, *asd*, *mip*, *momp*S, *pro*A, *neu*A	Time (year)	Site[a]	No. of strains
1,4,3,1,1,1,1	2003	a1, b1, e1, f	43
	2004	c	4
	2005	a1	11
	2006	b5	5
	2007	a2, b2, b3	3
	2008	a2, p/a2, a3, e2, e3, g	5
1,4,3,1,1,1,2	2003	b1	2
1,4,3,1,1,30,1	2006	b5	2
16,4,3,1,1,1,1	2008	a4	3
2,3,18,13,2,1,6	2006	b6	3
2,10,3,1,9,4,9	2003	b1	8
4,7,11,3,11,12,9	2004	d	5
11,14,16,16,15,13,2	2003	p/a1, b1	14
	2008	b4, e4	3

[a] a1, a2, a3, a4 = hospitals in Palermo city; b1, b2, b3, b4, b5, b6 = hotels in Palermo city; c = a hotel by the sea (east of Palermo); d = a hotel by the sea (west of Palermo); e1, e2, e3, e4 = private houses in Palermo city; f = a dentist's surgery in Palermo city; g = a Palermo city aqueduct (from a water supply in the country, east of Palermo); p/a1 = respiratory tract of an inpatient of a1; p/a2 = 202 respiratory tract of an inpatient of a2.

All these strains were typed by the standard SBT scheme, by the additional sequencing of *neu*A to create the extended SBT scheme, and by PFGE. Isolation, identification and standard SBT procedures were the same as those already described by Chiarini et al. [8]. Amplification and sequencing of *neu*A were performed, at the annealing temperature of 50 °C, with the forward (5′-CCGTTCAATATG GGGCTTCAG-3′) and reverse (5′-CGATGTCGA TGGATTCACTAATAC-3′) primers designed by Ratzow et al. [5]. PFGE was performed as described by Schoonmaker et al. [12] with slight modifications. Briefly, all isolates were digested with 20 U of the restriction enzyme SfiI (New England Biolabs, Beverly, MA, USA). Resulting fragments were separated by electrophoresis in 1% agarose gels with a CHEF Mapper apparatus (Bio-Rad Laboratories, Hercules, CA). Running conditions were 5 V/cm for 24 h at 14 °C

with switch times of 5 s (initial) and 35 s (final). Gels were stained with ethidium bromide, photographed under UV illumination and scanned in a Geldoc instrument (Bio-Rad). According to the criteria established by Tenover et al. [13] to define the pulsed-field clusters, 2—3 fragment differences were considered consistent with a single genetic event, and isolates with 0, 1, 2 or 3 genetic differences were considered indistinguishable, closely related, possibly related, or unrelated, respectively. Simpson's index of diversity (*D*), which takes into account the number of types present, as well as their relative abundance, was used to compare the discriminatory powers of standard SBT, extended SBT and PFGE [14].

Results

When typed by the standard six-gene SBT scheme, 73 of the 111 strains (65.8%) belonged to the prevailing SB type 1,4,3,1,1,1; 17 (15.3%) to the SB type 11,14,16,16,15,13 and 21 (18.9%) to additional five different SB types. Based upon these findings and the time-space allocation of *L. pneumophila* sg.1 isolates, a total of 26 likely epidemiologically unrelated groups were recognized: 17 groups (65.4%) belonged to the SB type 1,4,3,1,1,1; four (15.4%), to the SB type 11,14,16,16,15,13,2; five (19.2%), to the remaining five different SB types. One of the two clinical isolates (p/a2, Table 1), the environmental strains circulating in the hospital (a2, in Table 1) where the patient p/a2 had been admitted, and the strains isolated from the Palermo city aqueduct, all belonged to the prevailing SB type 1,4,3,1,1,1. On the contrary, although the environmental strains detected in the hospital where the second patient had been admitted (a1 and p/a1, in Table 1) belonged also to the SB type 1,4,3,1,1,1, a different SB type characterized this last clinical isolate.

The extension of the SBT scheme with the addition of the *neu*A gene allowed only a further differentiation of two SB type 1,4,3,1,1,1 environmental strains isolated in 2003 (Table 1).

When submitted to PFGE analysis, strains belonging to the seven standard (six-gene) SB types presented more than seven fragment differences and, according to the Tenover et al. criteria, confirmed to be unrelated [12] (Fig. 1A). On the contrary, all SB type 1,4,3,1,1,1 strains were indistinguishable with the exception of the two strains of the extended SB type 1,4,3,1,1,1,2 and an additional strain isolated in 2008 from the private house e3 (Fig. 1B and Table 1). In all these three cases

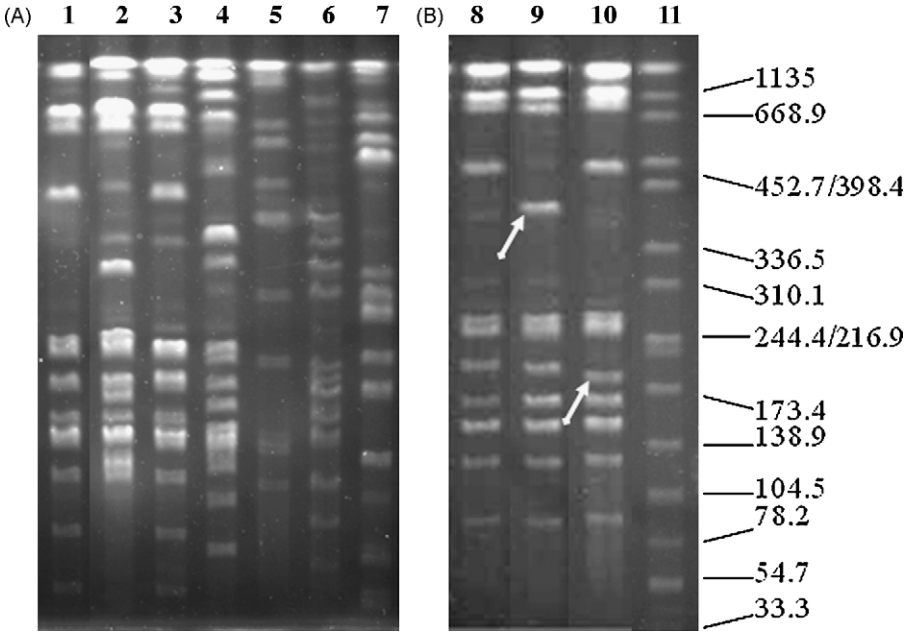

Figure 1 PFGE patterns of *L. pneumophila* strains belonging (A) to the seven genotypes detected in Northern Sicily by the standard SBT scheme, or differentiated (B) in the context of the prevailing SB type 1,4,3,1,1,1: lanes 1—7 = strains belonging to the standard SB types showed in the same order as in Table 1; lane 8 = a strain belonging to the extended, and still prevailing, SB type 1,4,3,1,1,1,1; lane 9: the strain belonging to the SB type 1,4,3,1,1,1,1 isolated in 2008 from the private house e/3; lane 10: a strain belonging to the SB type 1,4,3,1,1,1,2; lane 11 = *Salmonella* Braenderup H9812 DNA digested with XbaI. Arrows show, in lanes 9 and 10, products of DNA deletion from a lost larger fragment.

a single genetic difference was observed consisting in a deletion of DNA from a fragment of kb 201.02 in the first two strains, and of kb 441.3, in the third strain. According to the above interpretative criteria, due to their single genetic differences, these three strains should be considered as ''closely related'' to all the remaining 70 strains belonging to the extended SB type 1,4,3,1,1,1,1 that had been included into15 probably unrelated groups. In any case, their consequent classification — two strains by the extended SBT scheme or three strains by PFGE — would only increase the discriminatory power from a *D* value of 0.56 obtained by the standard SBT to the *D* values of 0.61 and 0.64, obtained with extended SBT and PFGE, respectively. All these values are much lower than the cut-off value of 0.90 that usually allows a typing result to be interpreted with confidence [13].

Discussion

Persistence over several years in the environment, in particular in the water supply, is a well known characteristic of some stable, predominant genotypes of *L. pneumophila* able to survive at low temperatures and in aquatic amoebae [15]. Persistence may also occur in huge water supplies, that allowed Oberdorfer et al. [16] to infer that in Heidelberg identical genotypes had been isolated from more than one hospital building, but not from buildings fed by different reservoirs.

In our study, both the single and the combined molecular approaches showed a low discriminatory power, which meant, when translated in terms of public health, a poor reliability of these methods as a field epidemiology tools. Indeed, in our setting attribution to different molecular types only is strongly predictive of a negative epidemiological link. On the contrary, detection of the same type in different isolates from environmental or human source needs to be interpreted in the light of the clonal circulation of a well defined molecular type and, consequently, cannot helpfully support the epidemiological investigations.

Detection of the predominant SB type 1,4,3,1,1,1,1 in a sample of water from an aqueduct supplying the city of Palermo further supports the hypothesis that the groups of the *L. pneumophila* sg.1 isolates, that we supposedly considered ''likely epidemiologically unrelated'' on the basis of their six-gene SB type and spatial/temporal context, could be actually ''strongly related''. Because the aqueduct water contamination could be responsible for the simultaneous, persistent presence of the same molecular type in many sites of the town, the possibility must be seriously considered that virtually all our isolates belonging to this type could be strongly related and, therefore, hardly distinguishable by any single or combined typing procedure. In such conditions, investigations of clinical cases and tracing to their environmental source require caution and support from sound ''traditional'' epidemiological data.

Conflict of interest

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